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ORIGINAL PAPER

Optimisation and validation of liquid chromatographic and partial least-squares-1 methods for simultaneous determination of enalapril maleate and nitrendipine in pharmaceutical preparations

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Simultaneous determination of enalapril maleate (ENA) and nitrendipine (NIT) in pharmaceutical preparations was performed using liquid chromatography (LC) and the partial least-squares-1 (PLS-1) method. In LC, the separation was achieved on a C8 column and the optimum mobile phase for good separation in a gradient elution programme was found to be acetonitrile–water (φ_r = 81 : 19) and optimum flow-rate, temperature, injection volume, and detection wavelength were set at 1.0 mL min^{-1}, 25 °C, 10 μL, and 210 nm, respectively. Dienogest was selected as an internal standard. In the spectrophotometry, a PLS-1 chemometric method was used. The absorbance data matrix related to the concentration data matrix was established by measurement of absorbances in their zero order spectra with an increment of $\Delta\lambda$ = 1 nm in the 220–290 nm range for ENA and with $\Delta\lambda$ = 1 nm in the 230–290 nm range for NIT in the PLS-1 method. Following this step, calibration was established by using this data matrix to predict the unknown concentrations of ENA and NIT in their binary mixture. These optimised methods were validated and successfully applied to a pharmaceutical preparation in tablet form and the results were subjected to comparison.

Keywords: enalapril maleate, nitrendipine, liquid chromatography, spectrophotometry, optimisation, validation

Introduction

Enalapril maleat (ENA), (2*S*)-1-[(2*S*)-2-{[(2*S*)-1-ethoxy-1-oxo-4-phenylbutan-2-yl]amino}propanoyl] pyrrolidine-2-carboxylic acid maleate, is an ACE inhibitor and it is used as an anti-hypertensive by decreasing the cleavage of angiotensin I to AngII and promoting reduction in bradykinin degradation (Santos et al., 2008; Lamontagne et al., 1995). Nitrendipine (NIT), (*RS*)-ethyl methyl 2,6-dimethyl-4-(3-nitrophenyl)-1,4-dihydropyridine-3,5-dicarboxylate (Fig. 1b), is a calcium-entry blocker and it is used as an anti-hypertensive by way of inhibiting calcium transport through the slow channel of the cardiac and vascular smooth muscle (Goa & Sorkin, 1987). The binary mixture of these drugs is widely used in the treatment of essential arterial hypertension.

In recent years, chemometric techniques in combination with the main separation techniques such as chromatographic or electrophoretic, i.e. multi-variate calibration techniques, have been applied to determination of the active ingredients in multi-component drugs interfered by excipients, which are added to pharmaceutical preparations in the process of their production (Palabiyik et al., 2008; Çağlayan et al., 2010; Sokullu et al., 2010; Karacan et al., 2011). These techniques are based on computer-aided instrumentation and algorithms and they are useful tools in resolving the overlapped spectra of mixtures of analytes when developing new analytical methods. One notable advantage of these techniques is the simultaneous analysis of the components in the mixture without any chemical pre-treatment or need to apply any graphical procedure on the spectra, such as deriva-

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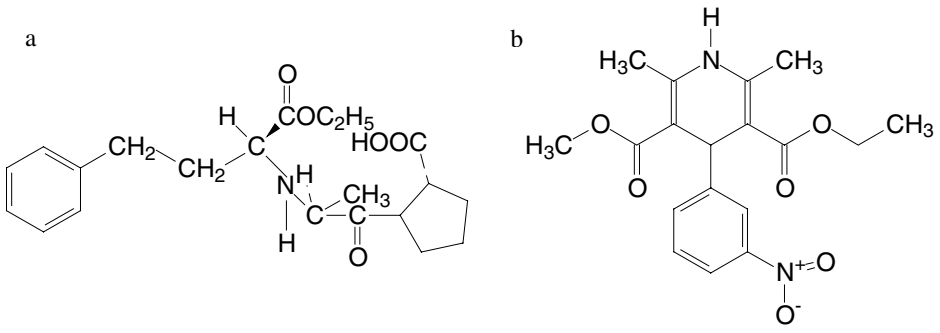

Fig. 1. Structures of enalapril maleate (a) and nitrendipine (b).

tive and ratio spectra spectrophotometry. They also provide a shorter analysis time, are cost-effective and require only simple instrumentation.

A survey of the literature was carried out and no reports concerning the simultaneous determination of ENA and NIT in pharmaceutical preparations were identified. The aim of this study was to develop a partial least-squares-1 method (PLS-1) as a chemometric technique in spectrophotometry and a liquid chromatographic method for the simultaneous determination of ENA and NIT in pharmaceutical preparations.

Experimental

Materials and methods

Enalapril maleate (Nobel İlaç Sanayi ve Ticaret A.Ş., Duzce, Turkey) and nitrendipin (Bayer Türk Kimya San. Ltd. Şti., Istanbul, Turkey) were used as the main components and dienogest (Shering–Plough Tıbbi Ürünler Ticaret A.Ş., Istanbul, Turkey) was used as an internal standard in the liquid chromatographic method. HPLC grade acetonitrile, methanol, and acetic acid were purchased from Sigma–Aldrich (Steinheim, Germany). Water for the mobile phase and for sample preparation in the liquid chromatographic method was supplied by the Purelab-UHQ (Elga Labwater, UK) water purification system. The mobile phase and the injection solution were degassed in an ultrasonic bath and filtered through a 0.45 μm nylon membrane prior to use. ENEAS® tablet (10 mg enalapril and 20 mg nitrendipine per tablet, batch No. B909) from Solvay İlaç Sanayi ve Ecza Tic. Ltd. Şti. (Istanbul, Turkey) was assayed as a commercial pharmaceutical preparation.

Shimadzu 1601 PC double-beam spectrophotometer with a fixed slit width (2 nm) connected to a computer loaded with Shimadzu UVPC software and a Standard quartz cuvette (10 mm) was used for the absorbance measurements obtained from this device.

In the PLS-1 method, Multi-variate Analysis Add-in for Excel v1.3 software (Brereton, 2002) was used.

A chromatographic system, Agilent Technologies HP 1100 (Germany), equipped with G1379A model degasser, G1311A quaternary pump, 61313A auto injector, and G1315B DAD detector was used. A C8 column (250 mm × 4.6 mm, 5 μm) (Phenomenex) was used. The chromatograms were recorded and the peaks quantified using an automatic integrator.

A pH meter (Hanna Instruments, HI221, Romania) was employed for pH measurement of water in the mobile phase. All solutions were degassed by ultrasonication in an ultrasonic bath (P Selecta, Barcelona, Spain), and filtered through a 0.45 μm nylon membrane prior to use., A heating magnetic stirrer (Velp Scientifica, ARE, Europe) was used for stirring solutions in some processes.

Solutions of ENA (204 μg mL^{-1}), NIT (204 μg mL^{-1}) and dienogest as internal standard (100 μg mL^{-1}) were prepared in an admixture of acetonitrile–water (pH was adjusted to 3.5 with acetic acid) ($\varphi_r = 81 : 19$) for the liquid chromatographic method. 0.45 mL of dienogest solution was added to each volumetric flask containing this binary mixture.

Solutions of ENA (500 μg mL^{-1}) and NIT (250 μg mL^{-1}) were prepared in methanol for the PLS-1 method.

For all the methods developed in this study, the contents of 10 tablets were accurately weighed and crushed in a mortar. An amount equivalent to one tablet was added to a 50 mL volumetric flask and diluted to volume with the acetonitrile–water admixture (pH was adjusted to 3.5 with acetic acid) ($\varphi_r = 81 : 19$) for the liquid chromatographic method and with methanol for the PLS-1 method, in individual volumetric flasks. After 10 min of stirring with a magnetic stirrer and 5 min of sonication in an ultrasonic bath, all the solutions were filtered through a 0.45 μm Whatman No. 42 filter paper into a 50 mL volumetric flask separately. For liquid chromatography, an appropriate amount of the filtrate and 0.45 mL of dienogest were transferred into a 25 mL volumetric flask and diluted with acetonitrile–water (pH was adjusted to 3.5 with acetic acid) ($\varphi_r = 81 : 19$) to the volume. This solution was injected into the chromatographic system. For the PLS-1 method, an appropriate amount of methanolic filtrate was transferred into a 25 mL volu-

metric flask and diluted to the volume with methanol. The absorbances of the solutions were measured at selected wavelengths.

Method optimisation and validation

In the liquid chromatographic method, acetonitrile content (%) in the mobile phase within a range of 60–85 % and pH of water within a range of 3.0–4.2 were investigated as factors affecting the responses obtained from the chromatographic system. System suitability tests including the retention factor, separation factor, resolution, asymmetry, number of theoretical plates, and relative standard deviations of the migration times were performed.

In order to establish optimum conditions for the PLS-1 method, absorbances from the UV spectrophotometric device were measured at different wavelengths within a range of 220–430 nm. Root-mean-square values were calculated using the chemometric software detailed in Materials and methods and these values were denoted as the markers for describing optimum conditions.

The linearity ranges were established using nine different concentrations in the range of 4.08–40.8 μg mL^{-1} for ENA and 0.82–61.2 μg mL^{-1} for NIT. Each solution was injected three times and the areas of the peaks for ENA and NIT measured at 210 nm were evaluated. The ratios of the peak areas of the investigated substances to that of the dienogest peak area (1.8 μg mL^{-1}) were calculated for each injection. Regression equations were established by plotting the ratio of the peak areas against the concentration of each substance. The linearity was evaluated by linear regression analysis, which was calculated by the least-squares regression.

For the PLS-1 method, a training set was established with 25 mixed solutions in methanol containing different concentrations of the active ingredients in the range of 0–40.0 μg mL^{-1} for ENA and 0–25.0 μg mL^{-1} for NIT.

For the liquid chromatographic method, LOQ (limit of quantification) and LOD (limit of detection) can be calculated by means of the following equations; $\mathrm{LOQ} = 10(\mathrm{SD}/m)$ and $\mathrm{LOD} = 3.3(\mathrm{SD}/m)$. In these equations, SD is the standard deviation of the response and m is the slope of the linearity range (International Conference on Harmonisation, 2003).

Figures-of-merit (FoM) were calculated for ENA and NIT (Kang et al., 2010).

The accuracy and precision were studied using three different solutions containing 12.0 μg mL^{-1}, 18.0 μg mL^{-1}, and 28.0 μg mL^{-1} of ENA and 6.0 μg mL^{-1}, 40.0 μg mL^{-1}, and 50.0 μg mL^{-1} of NIT for the liquid chromatographic method and 15.0 μg mL^{-1}, 20.0 μg mL^{-1}, and 30.0 μg mL^{-1} of ENA and 5.0 μg mL^{-1}, 10.0 μg mL^{-1}, and 15 μg mL^{-1} of NIT for the PLS-1 method. For repeatability studies, identical solutions were used for three days.

A chromatogram without ENA, NIT, and dienogest, i.e. the blank sample chromatogram, was evaluated to establish the selectivity and specificity as validation parameters. For describing these validation parameters in the PLS-1 method, the spectrum of bulk drugs and a commercial pharmaceutical preparation spectrum of the same amount as the bulk drugs were compared in terms of $\lambda_{\max}$ and spectrum shape.

Results and discussion

Method optimisation and validation

The solubility of NIT at different pH values is very low and almost unchanged. For that reason, changing the pH of water in the mobile phase did not have any distinctive effect on the retention time and peak shapes of NIT. Hence, NIT is considered as a hydrophobic compound; an increase in the percentage of acetonitrile in the mobile phase results in a decrease in the NIT retention time. As ENA has hydrophilic groups in its chemical structure and different solubility at different pH values, peak shape deteriorated with increasing pH values. A decrease in the pH of water in the mobile phase results in peak broadening. In addition, an increase in the pH of water results in a decrease in retention time of ENA.

Taking these results into account, the optimum mobile phase for good separation on a C8 column (250 mm × 4.6 mm, 5 μm particle) (Phenomenex) was found to be acetonitrile–water (pH was adjusted to 3.5 with acetic acid) ($\varphi_{\mathrm{r}} = 81 : 19$). The flow-rate, column temperature, injection volume and detection wavelength were set at 1.0 mL min^{-1}, 25 °C, 10 μL, and 210 nm, respectively. Dienogest was distinctly separated from ENA and NIT. For that reason, it was defined as the internal standard (IS).

Under these chromatographic conditions, ENA, dienogest (IS), and NIT peaks were well resolved and their retention times were found to be 2.65 min, 3.32 min, and 4.23 min, respectively. System suitability tests, including retention factor, separation factor, resolution, asymmetry, number of theoretical plates and relative standard deviations (%) of migration times, were carried out and the results are shown in Table 1. All of these results were acceptable within the limits as defined in the official guidelines as detailed in the Experimental section.

For the PLS-1 methods, the zero-order absorption spectra of ENA and NIT were overlapped in the range of 200–280 nm and this is shown in Fig. 2. Hence, the determination of ENA and NIT in their binary mixtures by measuring the absorbance at $\lambda_{\max}$ or any other wavelengths is not possible due to their mutual interference. To overcome this problem, the PLS-1 as a chemometric method was used in the simul-

Table 1. System suitability test results of ENA and NIT in liquid chromatographic method

Parameter	ENA	NIT
Retention factor (k')	0.89	2.02
Separation factor (α)	1.54	1.48
Resolution (R)	3.35	6.09
Asymmetry (A_s)	0.92	0.98
Number of theoretical plates (N)	2704	7157
Relative standard deviation (%) of migration times	0.32	0.14

Table 2. Training set used in PLS-1 method for ENA and NIT in admixture

Mixture No.	Solute concentration (μg mL^{-1})	
	ENA	NIT
1	20.0	10.0
2	40.0	10.0
3	5.0	2.5
4	40.0	2.5
5	15.0	25.0
6	40.0	5.0
7	20.0	25.0
8	15.0	10.0
9	30.0	2.5
10	40.0	15.0
11	30.0	25.0
12	20.0	15.0
13	40.0	25.0
14	5.0	25.0
15	5.0	15.0
16	20.0	2.5
17	30.0	15.0
18	15.0	15.0
19	5.0	5.0
20	15.0	2.5
21	20.0	5.0
22	0.0	10.0
23	20.0	0.0
24	10.0	20.0
25	20.0	10.0

taneous determination of these components in binary combination. In this technique, zero-order absorption spectra of the binary mixture of ENA and NIT were evaluated without any separation techniques required, which is an advantage of this method. First, a training set was established with 25 mL admixture solutions in methanol containing different concentrations of these active compounds (Table 2). Then, the optimum conditions for this method were investigated and a matrix of the absorbance data corresponding to the training set referred to above was constituted by the absorbance values measured at 71 wavelengths between 220–290 nm with an increment $\Delta\lambda = 1$ nm for ENA and at 61 wavelengths with an increment $\Delta\lambda = 1$ nm between 230–290 nm for NIT in their binary mixtures. We observed that good results were obtained by using non pre-processed data in the calculation procedures.

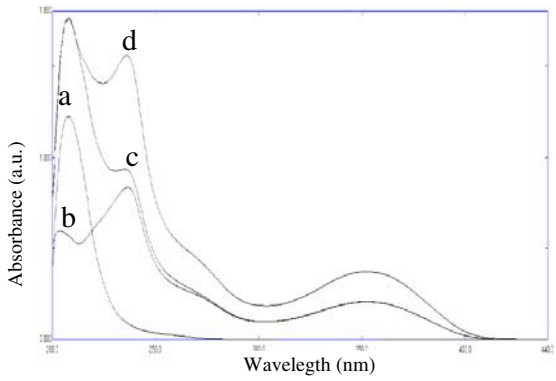

Fig. 2. Zero-order absorption spectra of 20 μg mL^{-1} ENA solution (a), 10 μg mL^{-1} NIT solution (b), solution containing 20 μg mL^{-1} of ENA and 10 μg mL^{-1} of NIT (c), and pharmaceutical preparation containing 9.6 μg mL^{-1} solution of ENA and 19.2 μg mL^{-1} solution of ENA in methanol (d).

To select the number of components for optimising the PLS-1, a cross-validation method, omitting one sample at a time, was used with evaluation of the training data sets. As a result, four latent variables for ENA and NIT were found to be optimum for the determination. Under these conditions, we observed that the RMS (Root-Mean-Squares) value was minimum and calculated as 0.22 and 0.32 for ENA and NIT, respectively. The predictive ability of the PLS-1 method is calculated as PRESS value (Predicted Residual Sum of Squares) and these are 3.11 and 0.29 for ENA and NIT, respectively.

Multivariate Analysis Add-in for Excel v1.3 software (Brereton, 2002) was used for all the PLS-1 calculations.

The linearity ranges were established as described in the Experimental section. The results for the liquid chromatographic method are given in Table 3.

For the PLS-1 methods, a training set was established with 25 mixture solutions in methanol containing different concentration of these active components with different concentration values in the range of 0–40.0 μg mL^{-1} for ENA and 0–25.0 μg mL^{-1} for NIT (Table 2).

For the liquid chromatographic method, LOQ and LOD values were calculated for each active component as described in the Method Optimisation and

Table 3. Statistical parameters obtained for liquid chromatographic method

Parameter	ENA	NIT
Linearity range (μg mL^{-1})	4.08–40.8	0.82–61.2
Slope of linearity range ± standard error (μg mL^{-1})	0.92 ± 0.0028	1.47 ± 0.0012
Intercept of linearity range ± standard error (μg mL^{-1})	–0.26 ± 0.0081	0.048 ± 0.0019
R	0.999	0.999

Table 4. Figures of merit for ENA and NIT in PLS-1 methods

Parameter	ENA	NIT
Sensitivity (SEN)	0.023	0.024
Analytical sensitivity (γ)	4.92	1.98
Effective resolution (γ^{-1})	0.20	0.51
Selectivity (SEL)	0.03	0.05
Signal-to-noise ratio (SNR)	12.13	10.07
Limit of detection (LOD) (μg mL^{-1})	0.61	1.52
Limit of quantification (LOQ) (μg mL^{-1})	2.01	5.01

Validation part. The LOQ values were calculated as 0.150 μg mL^{-1} and 0.022 μg mL^{-1} for ENA and NIT, respectively. The LOD values were determined as 0.05 μg mL^{-1} for ENA and 0.008 μg mL^{-1} for NIT.

Figures of Merit (FoM) were calculated as sensitivity (SEN), analytical sensitivity (γ), effective resolution (γ^{-1}), selectivity (SEL), signal-to-noise ratio (SNR), limit of detection (LOD), and limit of quantification (LOQ) for ENA and NIT in the PLS-1 method and results are shown in Table 4. These results indicate that simultaneous determination of ENA and NIT was achieved successfully with the training set (Table 2) described in the PLS-1 method.

The accuracy and precision were studied using three different solutions for ENA and NIT, as described in the Method Optimisation and Validation part; the results are given in Table 5 as mean recoveries and relative standard deviations (RSDs) with their standard errors. The high recovery values and low RSDs indicate that the liquid chromatographic and PLS-1 methods developed in this study could estimate the concentration values with perfect accuracy and a good precision of results intra-day and inter-day measurements. Standard errors of prediction values were

Table 5. Accuracy and precision data for ENA and NIT determinations

	Intra-day measurements			Inter-day measurements		
Added	Found[a]	Precision[b]	Accuracy[c]	Found[a]	Precision[b]	Accuracy[c]
(μg mL^{-1})	(μg mL^{-1})	RSD (%)	Bias (%)	(μg mL^{-1})	RSD (%)	Bias (%)
Liquid chromatographic method (ENA)						
12.00	12.30 ± 0.09	2.04	2.49	12.22 ± 0.04	1.64	–1.81
18.00	18.11 ± 0.07	1.15	0.06	18.05 ± 0.05	1.12	0.28
28.00	27.40 ± 0.05	0.60	–2.13	27.69 ± 0.06	1.22	–1.11
Liquid chromatographic method (NIT)						
6.00	5.98 ± 0.02	1.07	–0.25	5.99 ± 0.01	1.23	–0.11
40.00	38.80 ± 0.04	0.22	–2.99	38.92 ± 0.06	0.45	–2.69
50.00	48.95 ± 0.04	0.21	–2.09	49.12 ± 0.05	0.46	–1.77
PLS-1 method (ENA)						
15.00	14.60 ± 0.12	1.42	–2.68	14.60 ± 0.08	1.27	–2.68
20.00	20.16 ± 0.11	0.93	0.80	20.12 ± 0.09	1.08	0.62
30.00	30.62 ± 0.24	1.37	2.06	30.42 ± 0.17	1.39	1.40
PLS-1 method (NIT)						
5.00	5.08 ± 0.06	1.89	1.68	4.91 ± 0.05	2.45	–2.15
10.00	9.94 ± 0.11	1.92	–0.57	10.08 ± 0.06	1.50	0.55
15.00	14.81 ± 0.09	1.13	–1.26	14.79 ± 0.05	0.79	–1.38

a) Mean value ± standard error; *b*) relative standard deviation; *c*) (amount found – amount added)/amount added] × 100.

Table 6. Recovery results obtained from standard addition method for ENA and NIT in pharmaceutical formulation

Parameter	Liquid chromatography		PLS-1	
	ENA	NIT	ENA	NIT
Mean recovery (%)	100.04	100.70	107.17	101.73
Standard error	± 0.20551	± 0.18292	± 1.2911	± 0.47784
Confidence interval	0.36	0.32	2.75	0.96
RSD[a] (%)	0.79	0.70	2.69	1.15

a) Relative standard deviation.

Table 7. Assay results of commercial preparation (ENEAS® tablet (Batch No. B909) (labelled content of 10 mg ENA and 20 mg NIT per tablet)

Parameter	Liquid chromatography		PLS-1	
	ENA	NIT	ENA	NIT
Mean value ± standard error (mg)	10.23 ± 0.01	20.08 ± 0.02	10.28 ± 0.04	19.95 ± 0.04
RSD[a] (%)	0.23	0.20	0.81	0.45
Bias[b] (%)	2.26	0.42	2.81	–0.24
	ENA		NIT	
t-test[c]	1.09		1.89	

a) Relative standard deviation; *b*) (amount found – amount added)/amount added] × 100; *c*) theoretical value, $t = 2.31$ ($p = 0.05$).

calculated for the PLS-1 method and these were 0.35 and 0.03 for ENA and NIT, respectively.

Small changes in the combination of the mobile phase and pH of the water in the liquid chromatographic method did not cause any significant change in the analysis parameters. This indicated that the liquid chromatographic method developed was sufficiently robust for the simultaneous determination of ENA and NIT.

No interfering peak was observed in the blank chromatogram. Apart from this, there is no difference between the spectrum of bulk drugs and commercial pharmaceutical preparation spectrum with the same amount of the compounds under study as the bulk drugs (Fig. 2). These results indicated that the liquid chromatographic and spectrophotometric methods (PLS-1) thus developed were selective and specific for the simultaneous determination of ENA and NIT in their commercial preparations.

Analysis of pharmaceutical preparation

A recovery test was performed by the standard addition method to detect possible interferences from excipients in the pharmaceutical preparation which contained ENA and NIT, in accordance with the official validation guidelines, as described in the Method Validation part. In this test, known amounts of ENA and NIT were added to the crushed tablets in a 50 mL volumetric flask. Next, a similar procedure was performed to that described in the Method Optimisation and Validation part and the recovery values were calculated. The results are given in Table 6. The high recovery values indicate that there was no interference from the excipients in the tablet. Next, pharmaceutical preparation solutions were prepared in the manner as detailed previously in this study. Satisfactory results were obtained for each compound, and were found to be in agreement with the labelled content (Table 7). The results were obtained by the above methods and no significant difference was observed for the amount of drugs using the Student's *t*-test at the $p = 0.05$ level in commercial formulations.

Conclusions

In this study, liquid chromatographic and PLS-1 methods were developed for the simultaneous determination of ENA and NIT in their binary mixtures and in a pharmaceutical formulation in tablet form. Satisfactory results were obtained by these methods using only zero-order spectra in the procedures without the application of any other technique, such as derivative and ratio spectra and without the need for any prior separation procedure, these being the significant advantages of the PLS-1 method as a chemometric technique. However this method requires software for mathematical computations., The PLS-1 method developed in this study which uses spectrophotometric data is preferable to the liquid chromatographic method in terms of its simplicity and cost, without any time-consuming preparation procedures and using only methanol as solvent. Nevertheless, low LOD and LOQ values are the advantages of the liquid chromatographic method. All the statistical values in this study were within the acceptable limits. When the re-

sults obtained by the methods as described in this article were compared by applying Student t-test at $p = 0.05$ level, no significant difference was observed in the amount of active substances in the commercial formulations. Consequently,the liquid chromatographic and PLS-1 methods developed here have been proved to be sensitive, accurate, precise, selective and specific for application in routine analyses of ENA and NIT in pharmaceutical preparations.

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